

**Intrinsic Efficacy of Antipsychotics at Human D<sub>2</sub>, D<sub>3</sub>, and D<sub>4</sub>  
Dopamine Receptors: Identification of the clozapine metabolite N-  
desmethylozapine as a D<sub>2</sub>/D<sub>3</sub> partial agonist**

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## ABSTRACT

Drugs that antagonize D<sub>2</sub>-like receptors are effective antipsychotics, but the debilitating movement disorder side effects associated with these drugs cannot be dissociated from dopamine receptor blockade. The 'atypical' antipsychotics have a lower propensity to cause extrapyramidal symptoms (EPS) but the molecular basis for this is not fully understood, nor is the impact of inverse agonism upon their clinical properties. Using a cell-based functional assay we demonstrate that overexpression of G $\alpha$ o induces constitutive activity in the human D<sub>2</sub>-like receptors (D<sub>2</sub>, D<sub>3</sub> and D<sub>4</sub>). A large collection of typical and atypical antipsychotics was profiled for activity at these receptors. Virtually all were D<sub>2</sub> and D<sub>3</sub> inverse agonists, whereas none were D<sub>4</sub> inverse agonists, though many were potent D<sub>4</sub> antagonists. The inverse agonist activity of haloperidol at D<sub>2</sub> and D<sub>3</sub> receptors could be reversed by mesoridazine demonstrating that there were significant differences in the degrees of inverse agonism among the compounds tested. Aripiprazole, and the principle active metabolite of clozapine, N-desmethylozapine (NDMC) were identified as partial agonists at D<sub>2</sub> and D<sub>3</sub> receptors, although clozapine itself was an inverse agonist at these receptors. NDMC-induced functional responses could be reversed by clozapine. It is proposed that the low incidence of EPS associated with clozapine and aripiprazole use may be due, in part, to these partial agonist properties of NDMC and aripiprazole, and that bypassing clozapine blockade through direct administration of NDMC to patients may provide superior antipsychotic efficacy.

## Introduction

Blockade of dopamine receptors is the key mechanistic feature of antipsychotic medications believed to mediate many of their therapeutic benefits, but it is also responsible for many of the debilitating side effects associated with these drugs, particularly the extrapyramidal side effects (EPS) and hyperprolactinemia (Strange, 2001). The antipsychotics are divided into two major classes, the first generation D<sub>2</sub> blockers (henceforth typicals) and the second-generation serotonin/dopamine antagonists (henceforth atypicals). The typical antipsychotics, exemplified by drugs such as chlorpromazine and haloperidol, were the first generation of compounds used to treat schizophrenia, and tend to have uniformly high affinity for D<sub>2</sub> dopamine receptors, and produce a high incidence of EPS. There is a strong correlation between D<sub>2</sub> affinity, clinical dose, clinical efficacy and incidence of EPS for these agents (Creese et al, 1976; Seeman et al, 1976).

The atypical antipsychotics are distinguished by their lower incidence of EPS compared to the typical antipsychotics. As a group, the atypical drugs are much more heterogenous than the typical antipsychotics and thus it has been difficult to find a common mechanism of action explaining their distinct clinical profiles. The atypical drugs have varied affinities for D<sub>2</sub> receptors, and they produce a variety of side effects including metabolic disorders, weight gain, and cardiovascular effects. Many explanations have been proposed to explain the molecular basis of atypicality including affinity differences for D<sub>2</sub> receptors (Kapur and Seeman, 2001), 5HT<sub>1A</sub> agonism (Serretti et al, 2004), and selectivity for D<sub>3</sub> (Schwartz et al, 2000) and D<sub>4</sub> receptors (Jardemark et al, 2002). Further, all of the atypical antipsychotics are potent 5HT<sub>2A</sub> inverse agonists (Meltzer et al, 1989; Weiner et al, 2001).

Dopamine supersensitivity caused by chronic blockade of dopamine receptors has been proposed to explain the propensity of antipsychotics to cause EPS or tardive dyskinesia (TD) (Casey 2000). Although this hypothesis has concentrated on D<sub>2</sub> receptor occupancy, several antipsychotics are inverse agonists at D<sub>2</sub> receptors (Akam and Strange, 2004), and it has been demonstrated that inverse agonists cause recruitment and upregulation of G-protein coupled receptors (GPCRs) to the cell surface (Daeffler et al, 2000). Therefore, D<sub>2</sub> partial agonists may be particularly useful for treating schizophrenia because they would not upregulate dopamine receptor tone, but would still block the actions of dopamine at D<sub>2</sub> receptors (Tamminga and Carlsson, 2002). Consistent with this concept, aripiprazole, an atypical agent with partial agonist activity at D<sub>2</sub> receptors (Burriss et al, 2002), and D<sub>3</sub> receptors (Shapiro et al, 2003), in contrast to haloperidol, appears to have a low liability for inducing EPS/TD, does not elevate serum prolactin levels (Harrison and Perry, 2004), and does not upregulate either D<sub>2</sub> binding sites or D<sub>2</sub> mRNA (Inoue et al, 1997).

These findings emphasize the importance of defining efficacy, as well as the affinity of compounds for individual receptor subtypes to fully understand their molecular basis of clinical action. However, a comprehensive efficacy profile of compounds that target D<sub>2</sub>-like receptors is lacking. The impact of inverse agonism upon the clinical properties of these compounds is not fully understood, in part because the low sensitivity of many functional assays prevents reliable measurements of constitutive activity, and precludes functional assessment of receptors that signal poorly in heterologous systems such as D<sub>3</sub> (see Newman-Tancredi et al, 1999) and D<sub>4</sub> receptors (see Gazi et al, 2000; Yamaguchi et al, 1997; and Oldenhof et al, 1998). Previously we have shown that overexpression of G-proteins increases assay sensitivity, and induces constitutive activity of GPCRs (Burstein et al, 1997). We now report that overexpression of G $\alpha$

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dramatically improves the dynamic range of D<sub>3</sub>- and D<sub>4</sub>-mediated functional responses, and induces constitutive activity in D<sub>2</sub>, D<sub>3</sub>, and D<sub>4</sub> receptors. A comprehensive pharmacological profile of compounds that target D<sub>2</sub>-like receptors is described indicating that efficacy at D<sub>2</sub> is another factor that may affect certain clinical aspects of antipsychotic drug action.

## Methods

**Materials:** NIH-3T3 cells were from ATCC CRL 1658. O-nitrophenyl- $\beta$ -D- galactopyranoside and nonidet P-40 were from Sigma. Tissue culture media used was Dulbecco's modified Eagles medium (DMEM) (Gibco-BRL) supplemented with 4500-mg/l glucose, 4 nM L-glutamine, 50 U/ml penicillin G, 50 U/ml streptomycin and 10% calf serum. 96-well tissue culture dishes were from Falcon. Hanks balanced salt solution without magnesium chloride, magnesium sulfate, and calcium chloride, Trypsin-EDTA was all from Gibco-BRL.

**Drugs:** All compounds for R-SAT studies were solubilized as 10 mM stock solutions in either water or DMSO. Working dilutions were made from 50  $\mu$ M solutions in DMEM with 25% Ultraculture, 1% PSG. All compounds were obtained from Sigma/RBI (Natick, MA) except as follows: spiperone and remoxipride (Tocris, St. Louis, MO) tiospirone (Bristol Myers Squibb, Stamford, CT), chlorproethizene (Orgasynth Industries, Glasse, France), sultopride (IC Rom, Milan, Italy), moperone and bromperidol (Janssen Research Foundation, Beerse, Belgium), sertindole, trans-flupenthixol, and molindone (Lundbeck A/S, Copenhagen, Denmark), melperone (Cilag, Schaffhausen, Switzerland), while N-desmethylozapine (8-chloro-11-(1-piperaziny)-5H-dibenzo [b,e] [1,4] diazepine), sulforidazine, and 9-hydroxy-risperidone were synthesized by ACADIA Pharmaceuticals.

**Cell culture:** NIH-3T3 cells (ATCC no. CRL 1658) were incubated at 37 °C in a humidified atmosphere (5% CO<sub>2</sub>) in Dulbecco's modified Eagles tissue culture medium.

**Constructs:** The D<sub>2</sub> (short form) and D<sub>3</sub> receptors have been described (Weiner et al, 2001). The D<sub>3</sub> receptor contained a mutation (E275V), which was repaired by Quick-change mutagenesis<sup>TM</sup> (Stratagene). The D<sub>4</sub> receptor used in this study (variant 4.2) was cloned by polymerase chain reaction using Pfu Turbo<sup>TM</sup> (Stratagene) using primers derived from the GenBank accession entry L12398. The ras/rap chimera used in these studies (ras/rap1B(AA)) has been described previously (Ma et al, 2004). Human Regulator of G-protein Signaling 1 (RGS1) was cloned by PCR using oligonucleotides derived from the GenBank accession entry XM\_042967. The adenylyl cyclase Type II (AC2) construct used in these studies has been described [Ma et al, 2004, generous gift of Dr. P. Ram]. The G $\alpha$ o construct was described previously (Jones and Reed, 1987). cDNAs encoding bovine  $\beta$ 1 and  $\gamma$ 2 were generous gifts from B. Simonds. All clones were subcloned into the pSI vector (Promega Corp.) and sequence verified before use.

**Functional assays:** RepSelector Amplification Technology (R-SAT<sup>TM</sup>) assays were performed as described (Ma et al, 2004) with the following modifications. Briefly: Cells were plated one day before transfection using 7x10<sup>3</sup> cells in 0.1 ml of media per well of a 96-well plate. Cells were transiently transfected with 5 ng/well of receptor DNA, 20 ng/well ras/rap1B(AA), 2 ng/well AC2 and 30 ng/well pSI- $\beta$ -galactosidase (Promega, Madison, WI) per well of a 96-well plate using Polyfect (Qiagen) according to the manufacturers instructions. The use of ras/rap1B(AA) and AC2 was found to enable responses of G<sub>s</sub> and G<sub>i</sub>-coupled GPCRs in this functional assay (Ma et al, 2004). Where indicated, 5 ng/well each of G $\alpha$ o, G $\beta$ 1, G $\gamma$ 2 and RGS1 were co-transfected. The beta-gamma subunits G $\beta$ 1 and G $\gamma$ 2 were co-transfected to enhance the effects of G $\alpha$ o (Fishburn et al, 1999). One day after transfection media was



changed and cells were combined with ligands in DMEM supplemented with 25% Ultraculture synthetic supplement (Cambrex, Walkersville, MD) instead of calf serum to a final volume of 200  $\mu$ l/well. After five days in culture  $\beta$ -galactosidase activity was measured. The media were aspirated from the wells and the cells rinsed with phosphate buffered saline (PBS), pH=7.4. 200  $\mu$ l of PBS with 3.5 mM O-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) and 0.5% nonidet P-40 (both Sigma, St. Louis, MO) was added to each well and the 96-well plates were incubated at room temperature. After 3 h the plates were read at 420 nm on a plate-reader (Bio-Tek EL 310 or Molecular Devices). All data were analyzed using the computer programs Excel Fit and GraphPad Prism software (San Diego). Data for inverse agonism are reported as negative log values ( $pIC_{50}$ ). Functional antagonist  $IC_{50}$  data were adjusted for agonist occupancy using the Cheng-Prusoff equation  $K_i = IC_{50} / \{ 1 + [agonist] / EC_{50}^{agonist} \}$  to derive  $K_i$  values.

**Binding Studies:** Binding studies were carried out with [ $^3$ H]-raclopride ( $D_2$  and  $D_3$ ) (87 Ci/mmol, Amersham Pharmacia Biotech, Buckinghamshire, England) and [ $^3$ H]-spiperone ( $D_4$ ) (98 Ci/mmol, Amersham Pharmacia Biotech, Buckinghamshire, England) using membranes of NIH-3T3 cells transiently transfected as described above with  $D_2$ ,  $D_3$ , or  $D_4$  (10  $\mu$ g ea./15-cm dish) either with or without  $G\alpha_o$  (10  $\mu$ g) and prepared as described previously (Ma et al, 2004) using increasing concentrations of radiolabeled ligand for saturation binding experiments. Binding reactions were terminated by filtration through Type B glass fiber filters (Millipore, MultiScreen Harvest plates) presoaked for 30 min in 0.1% polyethyleneimine. Nonspecific binding was determined using 1  $\mu$ M haloperidol ( $D_2$  and  $D_3$ ) or 1  $\mu$ M spiperone ( $D_4$ ).

## Results

Using a functional assay based on cellular proliferation (R-SAT<sup>TM</sup>, Receptor Selection and Amplification Technology, see Ma et al, 2004), we have shown that overexpression of G-proteins augments both agonist-induced, and constitutive responses of GPCRs, and enables detection of inverse agonism (Burstein et al, 1997). In the presence of the D<sub>2</sub>/D<sub>3</sub>/D<sub>4</sub> -agonist pergolide, the D<sub>2</sub>-dopamine receptor mediates robust agonist responses, however, only weak responses of D<sub>3</sub> and no significant response of D<sub>4</sub> to pergolide were observed (**Figure 1 & Table 1**), consistent with earlier findings that D<sub>3</sub> and D<sub>4</sub> receptors signal poorly in heterologous systems (discussed above). Through reconstitution of receptor/G-protein interactions, it has been shown that D<sub>2</sub>-like receptors preferentially couple to G $\alpha$ o (Nickolls and Strange, 2004). When G $\alpha$ o was co-expressed, there was a dramatic increase in the potency and efficacy of pergolide at D<sub>2</sub>, and in the constitutive activity of D<sub>2</sub>. The constitutive response of D<sub>2</sub> induced by G $\alpha$ o could be reversed by haloperidol demonstrating that haloperidol acts as an inverse agonist at D<sub>2</sub> (**Figure 1B**). A small component of the constitutive response could not be reversed by haloperidol and likely represents non-receptor regulated interactions of G-protein with endogenous cellular components as observed previously (Burstein et al, 1997). The haloperidol-sensitive constitutive activity was not due to endogenous ligands present in the media as it was readily observed using synthetic media or in the presence of heterologous co-expression of the human dopamine transporter (data not shown). Similarly, overexpression of G $\alpha$ o induced a dramatic increase in the potency and efficacy of pergolide at D<sub>3</sub>, and significant responses to D<sub>4</sub> could now be detected (see **Figure 1C & 1E**, respectively; **Table 1**). G $\alpha$ o also induced robust increases in constitutive activity of D<sub>3</sub> and D<sub>4</sub>, however only constitutive activity of D<sub>3</sub> and not D<sub>4</sub> was reversed by haloperidol (**Figure 1D & 1F**).

Regulator of G-protein Signaling (RGS) 1 is a protein that selectively accelerates the GTPase activity of  $G\alpha_o$ , terminating its actions (Hoffman et al, 2001). To confirm that the observed increases in constitutive activity were due to increased receptor activation of G-proteins, we co-expressed RGS1 and  $G\alpha_o$  with  $D_2$ ,  $D_3$  or  $D_4$ . RGS1 reduced the potency of pergolide, and completely reversed the constitutive activity induced by  $G\alpha_o$  in all cases (**Figure 1, Table 1**).

Although increases in the level of expressed receptors could result in increased constitutive activity, co-expression of  $G\alpha_o$  had little or no effect on the expression levels of the receptors (**Table 1**). Thus the observed constitutive activity is due to the increased levels of  $G\alpha_o$ , and not because of changes in receptor levels.

We screened forty typical and atypical antipsychotics at the  $D_2$ ,  $D_3$ , and  $D_4$  receptors co-expressed with  $G\alpha_o$  and measured the functional responses, assigning a value of 100% to the basal response. Nearly all ligands tested were inverse agonists at  $D_2$  and  $D_3$ , reducing the response to between 50% and 70% of the basal response in most cases (**Figure 2A, 2B, Table 2**). Several compounds, including olanzapine, quetiapine, mesoridazine, and thioridazine, displayed substantially lower degrees of inverse agonist activity compared with haloperidol. Although virtually all of the antipsychotics tested were inverse agonists, only the primary active metabolite of clozapine, N-desmethylclozapine (henceforth NDMC), and aripiprazole were found to have agonist activity at  $D_2$  and  $D_3$ , increasing the response 2- to 3-fold over baseline at both receptors. None of the tested antipsychotics displayed significant agonist or inverse agonist activity at  $D_4$  (**Figure 2C, Table 2**), although many were potent antagonists (see below). In addition, none of these compounds displayed significant agonist or inverse agonist activity at cells transfected with

G-proteins alone, or at cells transfected with unrelated receptors (Burstein et al, unpublished observations).

To confirm that the differences in efficacy noted between partial agonists, partial inverse agonists, and full (with respect to haloperidol) inverse agonists were significant, D<sub>2</sub> and D<sub>3</sub> receptors co-expressed with G $\alpha$ o were challenged either with NDMC, haloperidol, mesoridazine, NDMC and mesoridazine, or NDMC and haloperidol. As shown in **Figure 3**, mesoridazine was able to block the inverse agonist activity of haloperidol, and the partial agonist activity of NDMC.

All of the compounds identified as having substantial inverse agonist activity (defined as less than -30%) were analyzed in full concentration response experiments at D<sub>2</sub> and D<sub>3</sub> (**Figure 4**) and all compounds, regardless of their activities as inverse agonists, were tested as antagonists of pergolide-induced activity at D<sub>2</sub>, D<sub>3</sub>, and D<sub>4</sub> (**Table 2**). The IC<sub>50</sub> values for reversal of G-protein induced constitutive activity at D<sub>2</sub> and D<sub>3</sub> and the K<sub>i</sub> values for reversal of agonist induced activity at these receptors were in general, very similar. Many of the tested ligands were potent functional antagonists at D<sub>4</sub>, despite the fact that they lack inverse agonist activity at D<sub>4</sub>. The vast majority of compounds displayed limited selectivity between D<sub>2</sub> and D<sub>3</sub> receptors, but relatively strong selectivity for D<sub>2</sub> and D<sub>3</sub> over D<sub>4</sub>. A notable exception was clozapine, which displayed 3- to 10-fold selectivity for D<sub>4</sub> over D<sub>2</sub> and D<sub>3</sub>, although NDMC was not selective for D<sub>4</sub>.

Aripiprazole and NDMC, which were the only compounds with agonist activity identified in the screen (see **Figure 2**), were further analyzed in full concentration response experiments. Aripiprazole was a potent partial agonist at both D<sub>2</sub> and D<sub>3</sub> (EC<sub>50</sub> = 0.1 and 1 nM, respectively), with about 60% efficacy relative to pergolide in each case (see **Figure 5**). Compared to

aripiprazole, NDMC was found to be a less potent, less efficacious agonist at both D<sub>2</sub> (20 nM, 35%) and D<sub>3</sub> (30 nM, 50%). Both aripiprazole and NDMC also functionally antagonized pergolide-induced activation of D<sub>2</sub> and D<sub>3</sub> (see **Table 2**). In comparison, the partial agonist S-(-)-PPP had significantly higher efficacy than aripiprazole at both D<sub>2</sub> and D<sub>3</sub> (Burstein et al, unpublished observations). Neither aripiprazole nor NDMC displayed appreciable agonist or inverse agonist activity at D<sub>4</sub> (not shown), although NDMC was a moderately potent antagonist at D<sub>4</sub> (**Table 2**). Haloperidol could block D<sub>2</sub> and D<sub>3</sub> responses to both NDMC (**Figure 6**) and aripiprazole (not shown). Similarly, clozapine blocked D<sub>2</sub> and D<sub>3</sub> responses to NDMC, demonstrating these compounds have opposing actions at these receptors (**Figure 6**).

## Discussion

These studies have provided a comprehensive pharmacological profile of an array of antipsychotics and related compounds at the human D<sub>2</sub>, D<sub>3</sub>, and D<sub>4</sub> dopamine receptors. Overexpression of G $\alpha$ o induced constitutive activation of these receptors, facilitating detection of both agonism and inverse agonism. Virtually all antipsychotics were D<sub>2</sub> and D<sub>3</sub> inverse agonists, whereas none were D<sub>4</sub> inverse agonists, though many were potent D<sub>4</sub> antagonists. A wide range of inverse agonist activities was observed, from high inverse agonist activity (e.g. haloperidol, pimozide, etc.) to very low inverse agonist activity (e.g. mesoridazine, quetiapine). Surprisingly the major active metabolite of clozapine, NDMC, like aripiprazole, was a partial agonist at D<sub>2</sub> and D<sub>3</sub> receptors.

Inverse agonism at D<sub>2</sub>-like receptors was first noted for haloperidol, which increased prolactin production and cyclic AMP formation in GH4C1 cells transfected with D<sub>2</sub>, and from GH3 cells expressing D<sub>2</sub> endogenously (Nilsson and Eriksson, 1993). Subsequently other D<sub>2</sub> antagonists, including several antipsychotic drugs, were identified as D<sub>2</sub> inverse agonists (Akam and Strange, 2004). Haloperidol, fluphenazine, and chlorpromazine were described as D<sub>3</sub> inverse agonists (Griffon et al. 1996). Other studies have reported mixed findings. Several D<sub>2</sub>-antagonists including haloperidol, raclopride, and clozapine were reported to be inverse agonists at D<sub>3</sub> but neutral antagonists at D<sub>2</sub> (Malmberg et al. 1998). In contrast, a series of D<sub>2</sub> antagonists were tested at D<sub>2</sub> and D<sub>4</sub> and all were reported to be neutral antagonists (Gilliland and Alper, 2000). A comprehensive functional profile of dopaminergic compounds at D<sub>2</sub>, D<sub>3</sub>, and D<sub>4</sub> subtypes, addressing inverse agonism, has not previously been available.

Compared with D<sub>2</sub>, the effect of expressing G $\alpha$ o on functional responses was greater for D<sub>3</sub> (see **Figure 1**), possibly reflecting the low efficiency of D<sub>3</sub> signaling previously observed

(Newman-Tancredi et al, 1999). Our results show that D<sub>3</sub> may have a greater requirement than most Gi/o-coupled receptors for G $\alpha$ <sub>o</sub>, which is highly expressed in brain, but is expressed at much lower levels in peripheral tissues, and in many commonly used cell lines (Asano et al, 1992).

Despite these differences in signaling, a strikingly similar pharmacology was observed at D<sub>3</sub> compared with D<sub>2</sub>. All compounds identified as inverse agonists at D<sub>2</sub> were also inverse agonists at D<sub>3</sub>, and the degree of inverse agonism for most of these compounds was very similar at D<sub>2</sub> and D<sub>3</sub>. NDMC and aripiprazole were both D<sub>3</sub> partial agonists. Finally, the potencies of all the compounds examined were similar at D<sub>2</sub> and D<sub>3</sub>, strongly suggesting that affinity for D<sub>3</sub> receptors does not confer an atypical antipsychotic profile as previously proposed (Schwartz et al, 2000).

Compared to D<sub>2</sub> and D<sub>3</sub> receptors, the D<sub>4</sub> receptor behaved differently. No response to D<sub>4</sub> was observed without co-expression of G $\alpha$ <sub>o</sub>, consistent with previous reports documenting poor coupling (Gazi et al, 2000). Constitutive internalization of the receptor has been proposed to explain the poor coupling observed with D<sub>4</sub> (Oldenhof et al, 1998). The D<sub>4</sub> receptor exists in several isoforms distinguished by the number of sequence repeat elements contained within the third intracellular (i3) loop. Only D<sub>4.2</sub> was examined in this study and thus the possible functional differences between isoforms were not addressed. However no functional differences between D<sub>4</sub> isoforms have been reported. Co-expression of G $\alpha$ <sub>o</sub> induced a high degree of constitutive activity at D<sub>4</sub>, but neither haloperidol, nor any of the other tested ligands were able to act as inverse agonists although many were able to potently block pergolide-induced activity. Since no D<sub>4</sub> inverse agonists were identified, we cannot exclude the possibility that the constitutive activity observed is not D<sub>4</sub>-mediated. However, given that the constitutive activity is

greatly diminished in cells expressing  $G\alpha_o$  without  $D_4$  (not shown), and that RGS1 suppressed the basal activity and increased the fold-response to pergolide in cells expressing  $D_4$  and  $G\alpha_o$ , suggests that the observed constitutive activity was  $D_4$ -mediated.

The pharmacology observed for  $D_4$  also differed considerably. Most compounds had significantly lower pKi values at  $D_4$  than at  $D_2$  or  $D_3$  with thiothixene and raclopride displaying the greatest selectivity for  $D_2/D_3$  over  $D_4$  (**Table 2**). Based on their  $D_2/D_4$  selectivity, most of these compounds would not be predicted to occupy  $D_4$  at clinical doses. Octoclotheperin displayed the highest affinity for  $D_4$ , although it had no appreciable selectivity for  $D_4$ . Other than the  $D_4$  reference compound L-745,870, only tiapride, molindone and clozapine displayed even modest selectivity for  $D_4$ .  $D_4$  selectivity, and in particular, the relative selectivity of clozapine for  $D_4$  over  $D_2$  and  $D_3$  has been proposed to confer atypical profiles (Jardemark et al, 2002), however the data presented above suggest that  $D_4$  does not mediate the therapeutic effects of antipsychotic medications nor does selectivity for  $D_4$  predict atypicality, in agreement with previous results (Roth et al, 1995).

Propensity to produce EPS/TD and other movement disorders, side effects that have been previously ascribed to  $D_2$  receptor occupancy (Creese et al, 1976; Seeman et al, 1976), may also depend on efficacy at  $D_2$ . The dramatic clinical differences seen with aripiprazole and clozapine, which are distinguished even from other atypical antipsychotics by their much lower liability for producing EPS/TD, coupled with their unique *in vitro* profiles, suggests that partial agonism at  $D_2$  is a desirable feature of an antipsychotic drug.  $D_2$  partial agonists offer several potential advantages for treating schizophrenia (see Tamminga and Carlsson, 2002). With the appropriate level of intrinsic activity, a partial agonist might act as an agonist at presynaptic  $D_2$  receptors (' $D_2$  autoreceptors') where receptor reserve is high, and act as an antagonist at postsynaptic  $D_2$



receptors where receptor reserve is low (Meller et al, 1986). Such a drug could provide superior efficacy for negative symptoms, may display reduced propensity to cause extra-pyramidal side effects, and yet still block the hyperdopaminergic activity thought to cause positive symptoms.

Discovery of the unique clinical properties of clozapine demonstrated that it was possible to separate the therapeutic benefits of antipsychotics from their side effects. The realization of the clinically beneficial characteristics of clozapine spurred the development of second generation or "atypical" antipsychotic drugs. Clozapine is still considered distinct from other 'atypicals' with respect to its use in psychotic patients suffering from EPS/TD (Charfi et al, 2004), and in treatment-induced psychosis in patients with Parkinson's disease (The Parkinson Study Group, 1999). Clozapine is active in treatment-resistant patients (Kane et al, 1988) and demonstrates superior improvements over other antipsychotics in cognitive function (Hagger et al, 1993). Thus, we have sought to define other molecular properties, if any, which might be responsible for the unique clinical features of clozapine, but to date, we have not identified any obvious features that distinguish clozapine itself from other atypical agents.

Several molecular features distinguish NDMC from clozapine, and all other typical and atypical antipsychotics. Uniquely among antipsychotics, NDMC is a potent  $M_1$  muscarinic receptor agonist whereas clozapine is a potent antagonist at  $M_1$  receptors (Sur et al, 2003; Weiner et al, 2004; Davies et al, 2005). Similarly, we now show that NDMC is a  $D_2/D_3$  partial agonist, whereas clozapine, and all other antipsychotics except for aripiprazole, are  $D_2/D_3$  receptor inverse agonists. Activating muscarinic cholinergic neurotransmission is widely considered to be a viable approach to treating cognitive deficits in schizophrenia (Friedman 2004), and as discussed above, attenuating dopaminergic neurotransmission may actually worsen cognitive

deficits. Therefore, we propose that the  $M_1$ ,  $D_2$  and  $D_3$  partial agonist properties of NDMC may account, in part, for the unique biochemical and clinical aspects of clozapine pharmacotherapy.

NDMC is the primary metabolite of clozapine, and achieves average plasma concentrations approximately 60-80% of that observed for clozapine in humans (Perry et al, 1991). Therefore, during clozapine therapy, significant levels of both a  $D_2/D_3$  receptor inverse agonist (clozapine) and a  $D_2/D_3$  receptor partial agonist (NDMC) are present in substantial concentrations in most patients. We have observed that there is a positive correlation between superior clinical outcomes and the metabolism of clozapine to NDMC in schizophrenic subjects (Weiner et al, 2004). Specifically, higher ratios of NDMC to clozapine, and not the absolute levels of either compound alone, predicted better improvements in clinical response, including multiple measures of cognition and quality of life scores, suggesting that a pharmacological interaction between the two molecules may affect clinical efficacy. Given that clozapine blocks the agonist actions of NDMC at  $M_1$ ,  $D_2$  and  $D_3$ , the observations that improved clinical outcomes are positively correlated with increased NDMC/clozapine ratios suggests that overcoming clozapine blockade of these receptors with NDMC may confer some of the unique clinical benefits ascribed to clozapine pharmacotherapy. Inter-individual variation, and the complex pharmacodynamic relationships between clozapine and NDMC would be bypassed if NDMC were directly administered to patients. These observations, coupled with the  $D_2/D_3$  partial agonist properties of NDMC reported herein, add additional support to the hypothesis that NDMC may itself display superior antipsychotic activity. This possibility is currently being tested clinically.

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**Footnotes:**

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## Figure Legends

### **Figure 1. Effects of G $\alpha$ o and RGS1 on functional responses of D<sub>2</sub>, D<sub>3</sub>, and D<sub>4</sub> receptors.**

Plasmids encoding receptors (**R**), G $\alpha$ o (**G**), and RGS1 (**RGS**) were transfected into NIH-3T3 cells and functionally analyzed in the presence of the indicated concentrations of pergolide and haloperidol using the R-SAT functional assay as described in the methods. **A and B**) D<sub>2</sub>; **C and D**) D<sub>3</sub>; **E and F**) D<sub>4</sub>. Data points represent the mean of two separate determinations in each case.

**Figure 2. Functional screen of antipsychotics.** Receptors were co-transfected with G $\alpha$ o and functionally analyzed as described above in the presence of 1  $\mu$ M concentrations of the indicated drugs except for olanzapine, thioridazine, and N-desmethyloanzapine, which were tested at 300 nM to avoid non-receptor mediated cellular effects. The basal response in the absence of added ligand was assigned a value of 100% and all other responses were normalized to that value. **A**) D<sub>2</sub>. **B**) D<sub>3</sub>. **C**) D<sub>4</sub>. Values shown represent the mean of at least two or more independent experiments with eight individual determinations per experiment.

**Figure 3. Blockade of agonism and inverse agonism.** Individual dopamine receptors were co-transfected with G $\alpha$ o and functionally analyzed as described above in the presence of mesoridazine (500 nM, denoted Meso), or haloperidol (10 nM, denoted Haldol), or both (Mes+Hal). **A and C**) D<sub>2</sub>; **B and D**) D<sub>3</sub>. Responses were normalized to the response in the absence of added ligands, which was assigned a value of 100%. Shown are representative individual experiments. Data represent the means of 24 individual determinations. Statistical significance was assessed using the unpaired t-test. \*Significantly different (p<0.01) from

haloperidol (**A** and **B**) or mesoridazine (**C** and **D**) alone. <sup>#</sup>Significantly different ( $p < 0.01$ ) from no drug.

**Figure 4. Profiles of dopaminergic inverse agonists.** Respective dopamine receptors were co-transfected with G $\alpha$ o and functionally analyzed in the presence of the indicated concentrations of ligands using the R-SAT functional assay as described above. **A** and **B**) D<sub>2</sub>. **C** and **D**) D<sub>3</sub>. Data points represent the mean of two separate determinations in each case. Responses were normalized to the basal response in the absence of ligand, which was assigned a value of 100%.

**Figure 5. Profiles of dopaminergic agonists.** Respective dopamine receptors were co-transfected with G $\alpha$ o and functionally analyzed in the presence of the indicated concentrations of ligands using the R-SAT functional assay as described above. **A**) D<sub>2</sub>. **B**) D<sub>3</sub>. Data points represent the mean of two separate determinations in each case. Responses were normalized to the response to pergolide, which was assigned a value of 100%.

**Figure 6. Opposing actions of NDMC and clozapine.** Plasmids encoding D<sub>2</sub> or D<sub>3</sub> and G $\alpha$ o were transfected into NIH3T3 cells and functionally analyzed in the presence of the indicated concentrations of NDMC, or pergolide, or in the presence of 300 nM NDMC and the indicated concentrations of haloperidol and clozapine using R-SAT as described in the methods. Data points represent means of two separate determinations. **A**) D<sub>2</sub>. **B**) D<sub>3</sub>. Responses were normalized to the response to pergolide, which was assigned a value of 100%.

**Table 1.**

	<b>R</b>	<b>R+G</b>	<b>R+G+RGS</b>
<b>Agonist:</b>			
<b>D2</b>	1.8 ± 1.4	0.2 ± 0.2	1.0 ± 0.7
<b>D3</b>	0.7 ± 0.5	0.1 ± 0.1	0.5 ± 0.4
<b>D4</b>		8.4 ± 4.3	96 ± 84
<b>Inverse Agonist:</b>			
<b>D2</b>	-	0.8 ± 0.6	-
<b>D3</b>	-	0.5 ± 0.4	-
<b>D4</b>	-	-	-
<b>Expression:</b>			
<b>D2</b>	675 ± 21	690 ± 28	nd
<b>D3</b>	433 ± 128	486 ± 178	nd
<b>D4</b>	256 ± 16	164 ± 61	nd

**Table 1.** Effect of G $\alpha$ o and RGS1 on functional responses to D<sub>2</sub>, D<sub>3</sub>, and D<sub>4</sub> receptors.

Plasmids encoding the respective receptors, and where indicated G $\alpha$ o and RGS1 were transfected into NIH-3T3 cells and functionally analyzed in the presence of the serial dilutions of pergolide (Agonist) and haloperidol (Inverse Agonist) using the R-SAT functional assay as described in the methods. Data are reported in nM units as EC<sub>50</sub> and IC<sub>50</sub> for agonist and inverse agonist, respectively, and represent the means of four or more independent experiments in each case. To obtain expression levels, radioligand binding, using [<sup>3</sup>H]-raclopride (D<sub>2</sub> and D<sub>3</sub>) and [<sup>3</sup>H]-spiperone (D<sub>4</sub>) was carried out as described in the methods. A dash indicates no measurable response. nd = not done. Data represents the mean of two to three independent experiments in each case.

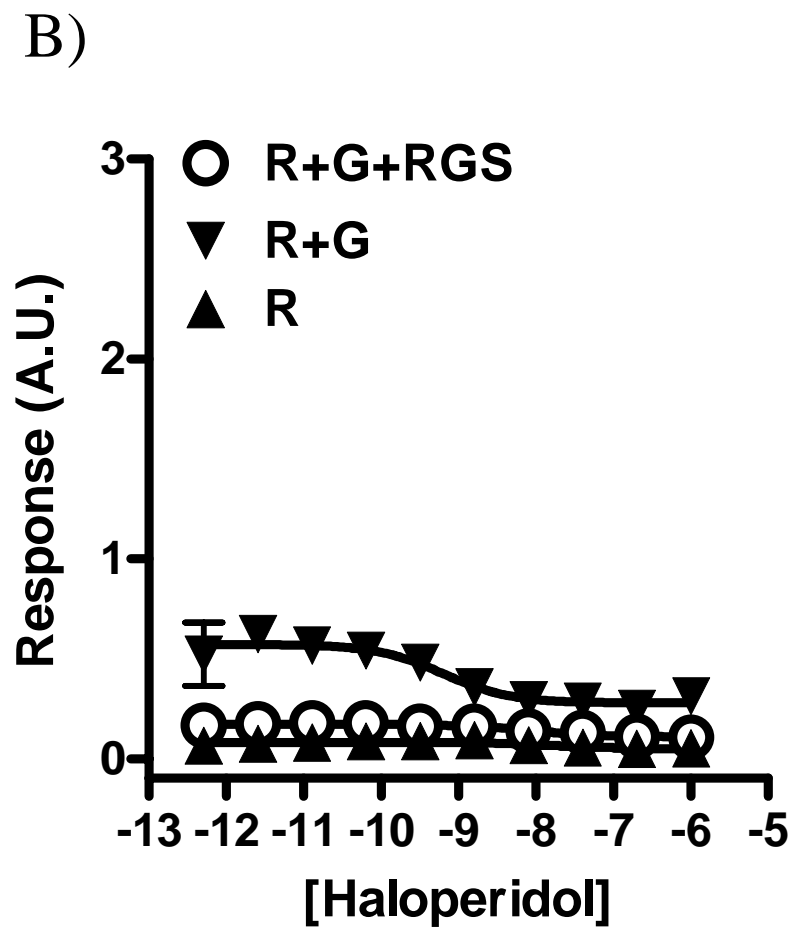
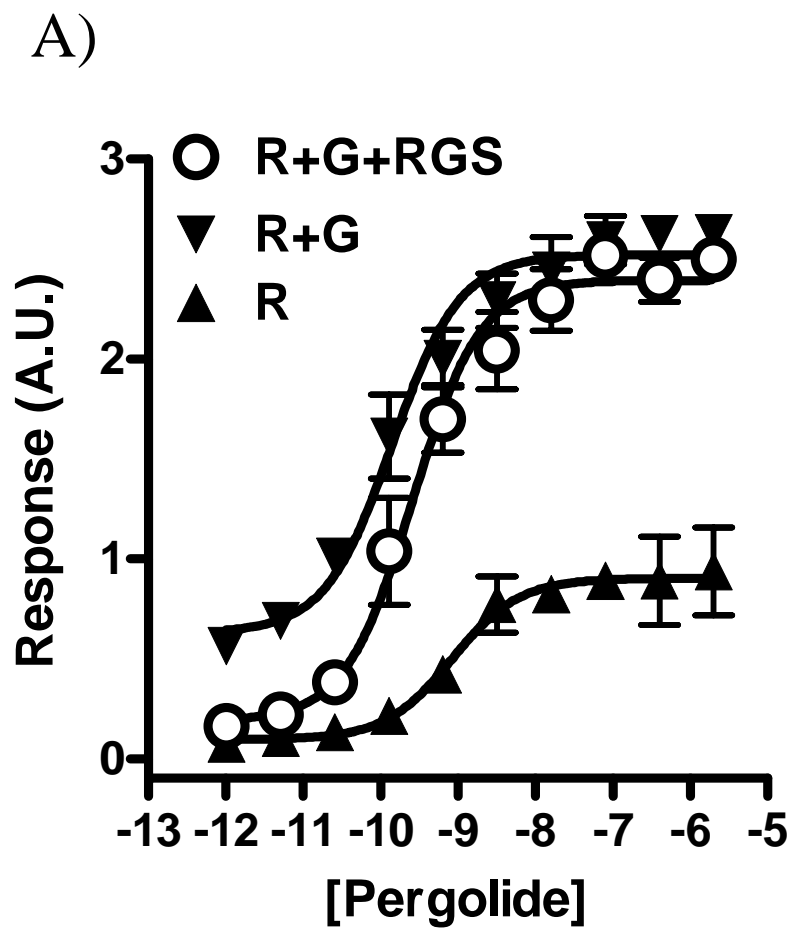
Table 2.

Ligands	D2			D3			D4	
	Response (%)	IC50	Ki	Response (%)	IC50	Ki	Response (%)	Ki
bromperidol	-54 ± 6	2.1 ± 0.6	1.0 ± 0.6	-57 ± 18	2.3 ± 0.7	0.2 ± 0.1	8 ± 8	48 ± 41
spiperone	-49 ± 1	0.3 ± 0.1	0.03 ± 0.01	-46 ± 6	0.2 ± 0.1	0.0 ± 0.0	8 ± 3	3.7 ± 1.7
fluspirilene	-48 ± 6	0.2 ± 0.1	0.2 ± 0.0	-55 ± 14	4.3 ± 0.4	0.4 ± 0.2	-1 ± 7	8.8 ± 4.7
pimozide	-45 ± 14	0.5 ± 0.1	2.4 ± 1.3	-40 ± 11	0.2 ± 0.2	0.3 ± 0.1	-1 ± 23	1.8 ± 1.5
prochlorperazine	-43 ± 16	0.2 ± 0.0	1.1 ± 0.4	-47 ± 10	2.4 ± 1.0	3.4 ± 2.5	-4 ± 7	1132 ± 516
haloperidol	-43 ± 15	0.8 ± 0.2	0.6 ± 0.3	-53 ± 12	0.6 ± 0.2	0.2 ± 0.1	-11 ± 2	22 ± 11
trifluoperidol	-42 ± 12	0.2 ± 0.2	0.4 ± 0.1	-46 ± 12	3.5 ± 2.1	4.2 ± 2.5	13 ± 3	326 ± 91
cisflupenthixol	-41 ± 7	0.2 ± 0.1	0.5 ± 0.2	-48 ± 14	2.9 ± 2.3	0.6 ± 0.5	17 ± 10	131 ± 82
sulpiride	-41 ± 4	11 ± 9	8.6 ± 1.9	-45 ± 13	8.2 ± 4.4	7.9 ± 3.8	16 ± 8	54 -
chlorproethazine	-40 ± 14	10 ± 5	11 ± 5	-37 ± 23	18 ± 9	5.9 ± 1.9	25 ± 9	23 ± 11
butaclamol	-40 ± 2	0.3 ± 0.3	0.4 ± 0.4	-41 ± 32	1.6 ± 1.0	1.7 ± 1.5	21 ± 5	162 ± 77
amoxapine	-40 ± 3	38 ± 6	3.6 ± 1.5	-32 ± 5	31 ± 9	11 ± 7	-8 ± 3	40 ± 35
fluphenazine	-39 ± 6	0.2 ± 0.1	0.4 ± 0.2	-45 ± 12	2.1 ± 0.3	1.4 ± 0.8	32 ± 3	7.1 ± 1.7
sertindole	-39 ± 4	2.7 ± 1.1	2.0 ± 0.3	-45 ± 23	12 ± 4	7.7 ± 1.9	3 ± 1	28 ± 14
ocaperidone	-38 ± 11	0.1 ± 0.0	0.4 ± 0.3	-49 ± 14	2.9 ± 1.9	0.7 ± 0.3	10 ± 5	6.6 ± 5.1
risperidone	-37 ± 3	0.3 ± 0.3	0.6 ± 0.3	-47 ± 26	2.4 ± 2.3	1.6 ± 1.3	15 ± 6	18 ± 7
remoxipride	-37 ± 3	16 ± 6	105 ± 38	-23 ± 5	70 ± 45	>300 -	7 ± 8	>1000 -
tiapride	-35 ± 10	31 ± 13	226 ± 223	-43 ± 10	324 ± 66	95 ± 66	7 ± 8	14 ± 10
ziprasidone	-34 ± 12	3.0 ± 1.1	5.7 ± 1.5	-36 ± 3	8.4 ± 5.8	7.3 ± 3.5	9 ± 4	>1000 -
raclopride	-34 ± 9	0.5 ± 0.3	2.4 ± 0.8	-39 ± 8	1.8 ± 0.9	1.2 ± 0.7	16 ± 5	nr -
moperone	-34 ± 7	1.0 ± 0.6	0.7 ± 0.2	-41 ± 15	1.0 ± 0.3	0.1 ± 0.1	14 ± 4	27 ± 15
tefludazine	-33 ± 2	0.3 ± 0.2	0.7 ± 0.2	-53 ± 17	1.7 ± 1.0	0.6 ± 0.3	8 ± 15	4.5 ± 2.2
transflupenthixol	-32 ± 6	17 ± 6	21 ± 6	-43 ± 16	226 ± 78	99 ± 67	12 ± 13	>1000 -
clozapine	-31 ± 10	71 ± 21	72 ± 20	-28 ± 7	27 ± 13	79 ± 28	14 ± 6	7.6 ± 4.4
molindone	-30 ± 8	3.8 ± 1.5	20 ± 7	-35 ± 10	94 ± 55	51 ± 17	13 ± 4	5.6 ± 5.3
chlorpromazine	-29 ± 14	1.7 ± 1.1	8.1 ± 2.1	-34 ± 19	3.0 ± 1.2	6.3 ± 4.8	33 ± 8	24 ± 5
sulforidazine	-29 ± 13	0.2 ± 0.0	0.3 ± 0.2	-33 ± 12	nd -	0.7 ± 0.2	nd -	nd -
9-OH-risperidone	-29 ± 13	1.0 ± 0.2	0.4 ± 0.4	-31 ± 11	0.9 ± 0.8	0.1 ± 0.1	nd -	nd -
thiothixene	-27 ± 15	0.2 ± 0.0	0.03 ± 0.01	-32 ± 20	0.3 ± 0.2	0.5 ± 0.4	14 ± 9	nr -
sultopride	-24 ± 6	4.5 ± 0.7	1.6 ± 1.0	-23 ± 5	nd -	3.8 ± 3.2	14 ± 3	nd 0.0
melperone	-24 ± 4	nd -	3.6 ± 0.5	-25 ± 1	13 ± 2	4.9 ± 4.6	11 ± 3	>1000 -
octoclothebin	-24 ± 6	0.2 ± 0.1	0.7 ± 0.3	-40 ± 11	0.9 ± 0.2	0.2 ± 0.0	17 ± 8	0.4 ± 0.4
promazine	-18 ± 13	nd -	>300 -	-15 ± 19	nd -	159 ± 76	23 ± 13	nr -
quetiapine	-14 ± 10	nd -	16 ± 12	-18 ± 15	nd -	9.2 ± 5.7	7 ± 8	nr -
mesoridazine	-14 ± 9	nd -	4.3 ± 1.4	-18 ± 10	nd -	2.6 ± 1.4	25 ± 1	9.1 ± 8.9
N-desmethyloanzapine	-14 ± 7	nd -	32 ± 29	4 ± 11	nd -	>300 -	10 ± 3	nd 0.0
olanzapine	-13 ± 12	nd -	106 ± 43	-13 ± 12	nd -	91 ± 47	27 ± 6	31 ± 24
thioridazine	-12 ± 15	nd -	21 ± 16	-19 ± 17	nd -	7.4 ± 5.9	8 ± 5	nr -
L745,870	nd -	nd -	343 ± 297	nd -	nd -	>1000 -	nd -	1.8 ± 1.5
aripiprazole	194 ± 42	nd -	4.4 ± 0.9	142 ± 30	nd -	3.1 ± 0.3	16 ± 8	>1000 -
N-desmethylclozapine	129 ± 21	nd -	89 ± 26	88 ± 20	nd -	153 ± 57	14 ± 7	70 ± 21

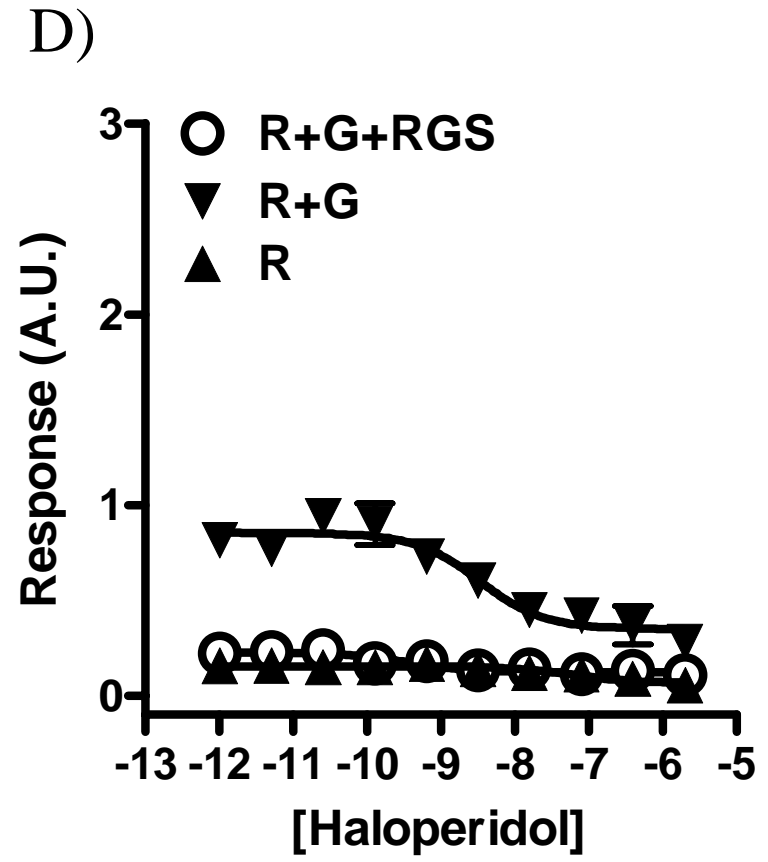
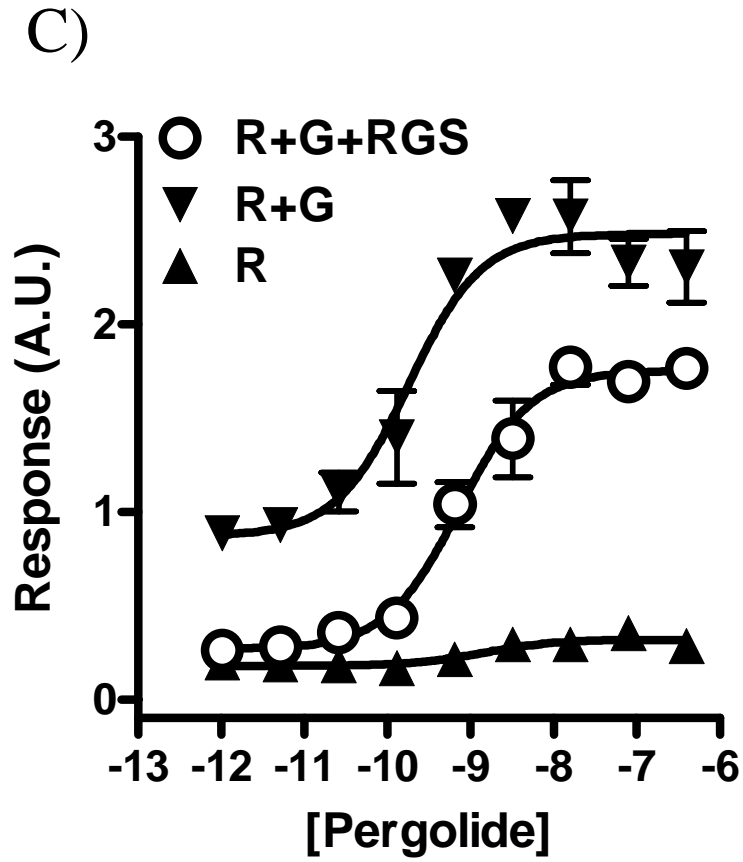
**Table 2.** Antagonist and inverse agonist profiles of dopaminergic ligands. For determination of intrinsic efficacy (%), D<sub>2</sub>, D<sub>3</sub>, and D<sub>4</sub> receptors were co-transfected with G $\alpha$ o and functionally analyzed using R-SAT as described above in the presence of 1  $\mu$ M concentrations of the indicated drugs except for olanzapine, thioridazine, and N-desmethyloanzapine, which were tested at 300 nM to avoid non-receptor mediated effects. Response is defined as [(drug response/basal response)\*100%]-100%. Thus a neutral antagonist would have a response equal to 0%. Values shown represent the mean of two or more independent experiments with eight individual determinations per experiment. To determine inverse agonist activity (reported in nM units as IC<sub>50</sub>), D<sub>2</sub> and D<sub>3</sub> receptors were co-transfected with G $\alpha$ o and functionally analyzed in the presence of serial dilutions of the indicated ligands. To determine antagonist activity (reported in nM units as Ki), D<sub>2</sub> receptors were transfected without G $\alpha$ o, and D<sub>3</sub> and D<sub>4</sub> receptors were transfected with both G $\alpha$ o and RGS1 to increase the dynamic range of the assay. Cells were incubated with a fixed concentration of pergolide (5x the previously determined EC<sub>50</sub> for each receptor) and serial dilutions of the indicated ligands. Ki values were calculated as described in the methods. nd = not done. nr = no response. Data represent the means of at least two or more independent experiments.



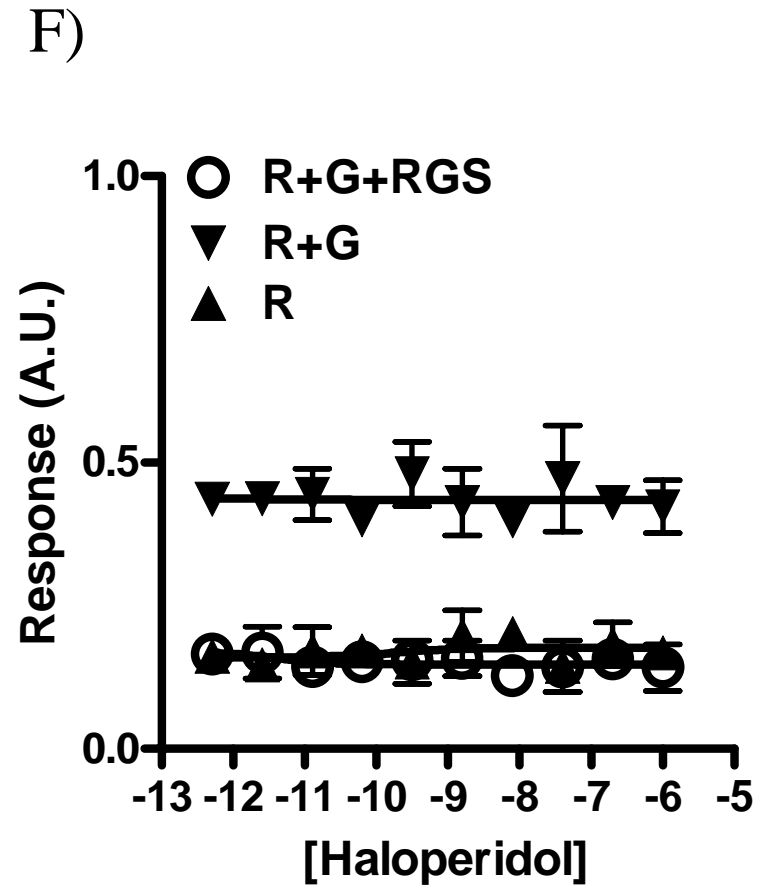
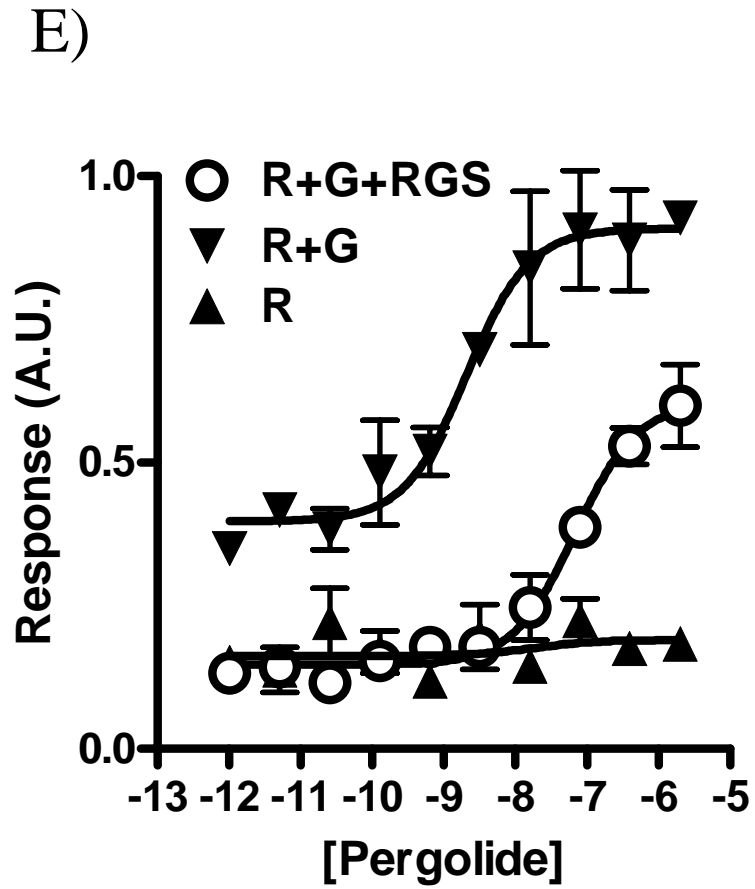
## Figure 1



## Figure 1



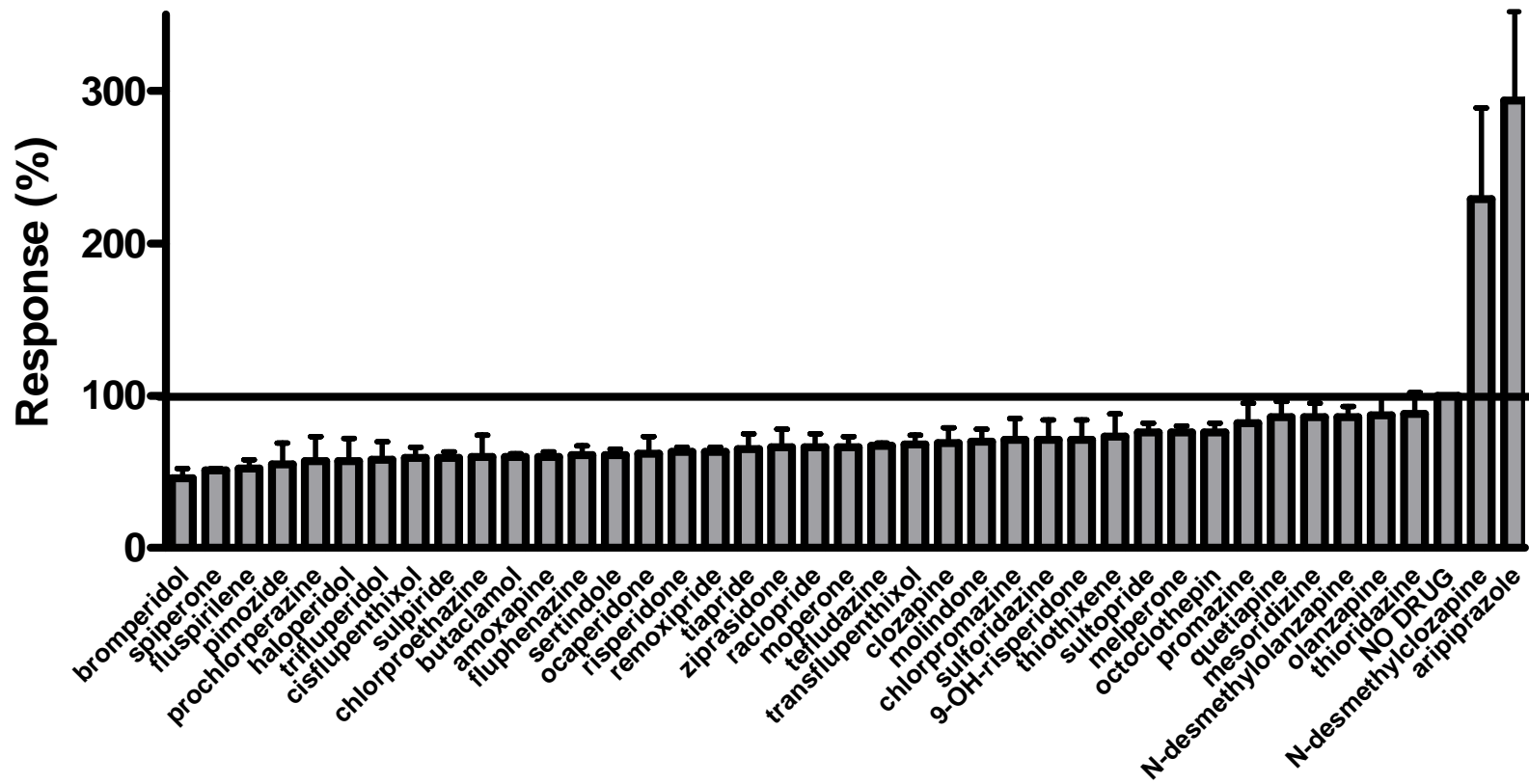
## Figure 1



# Figure 2

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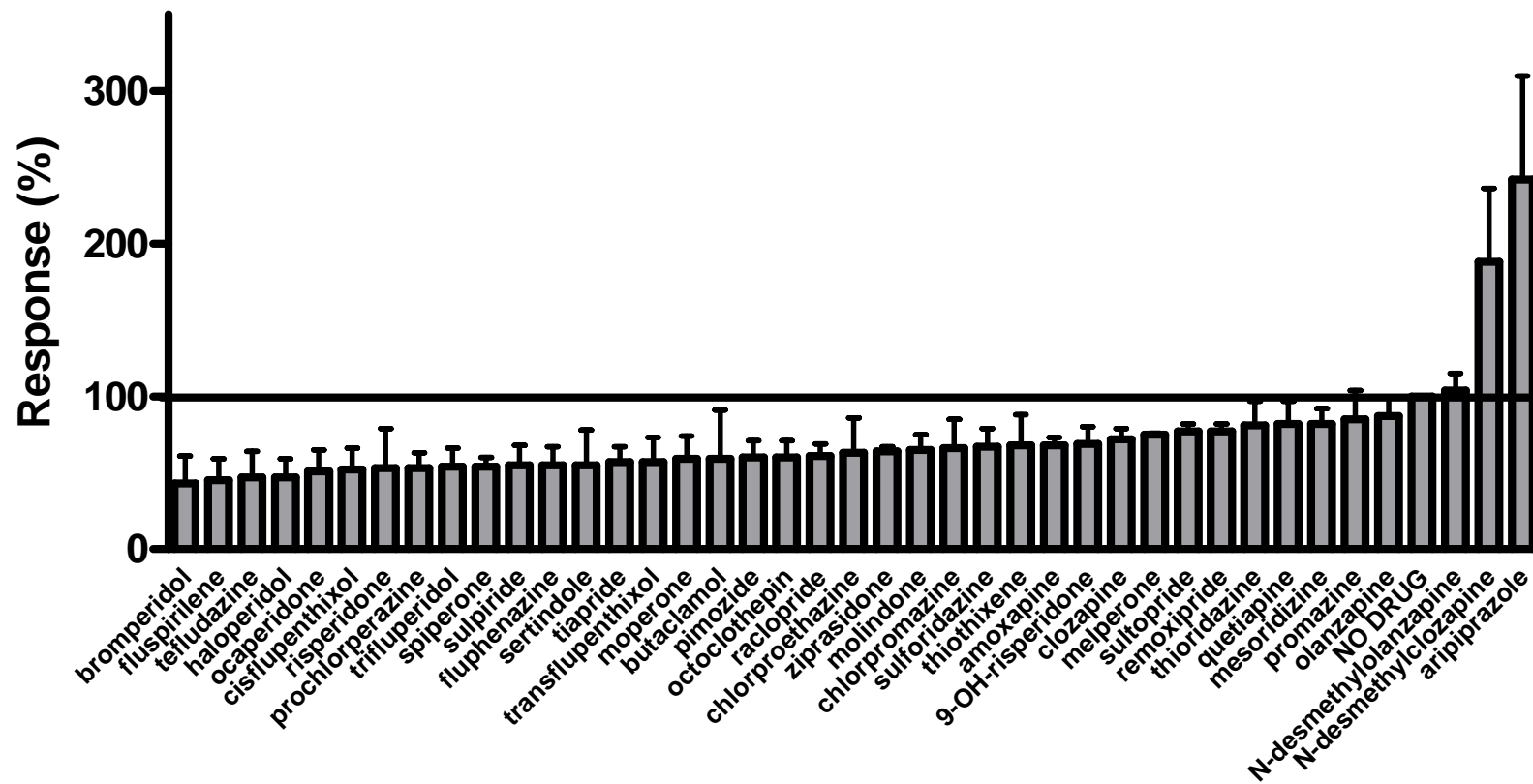
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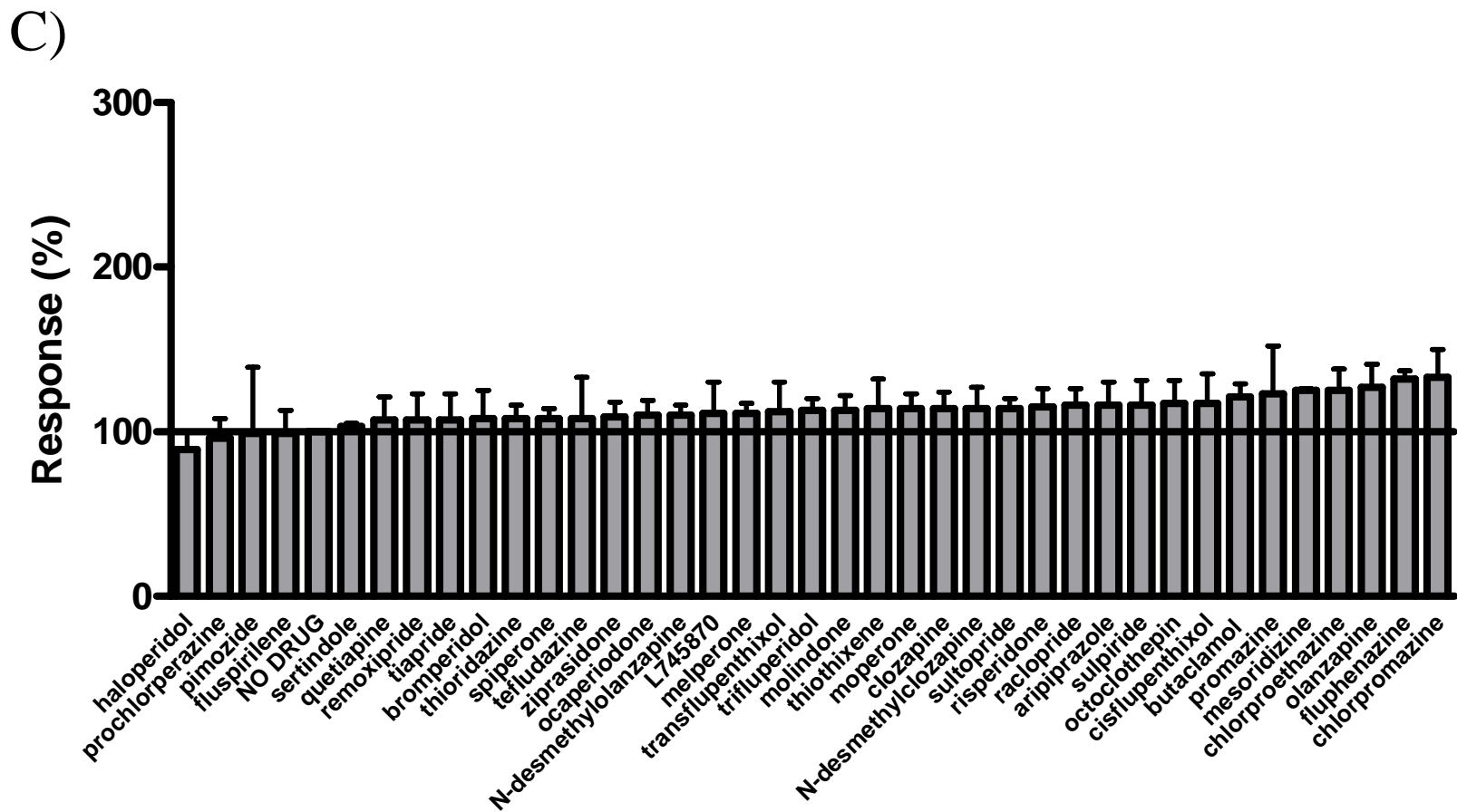
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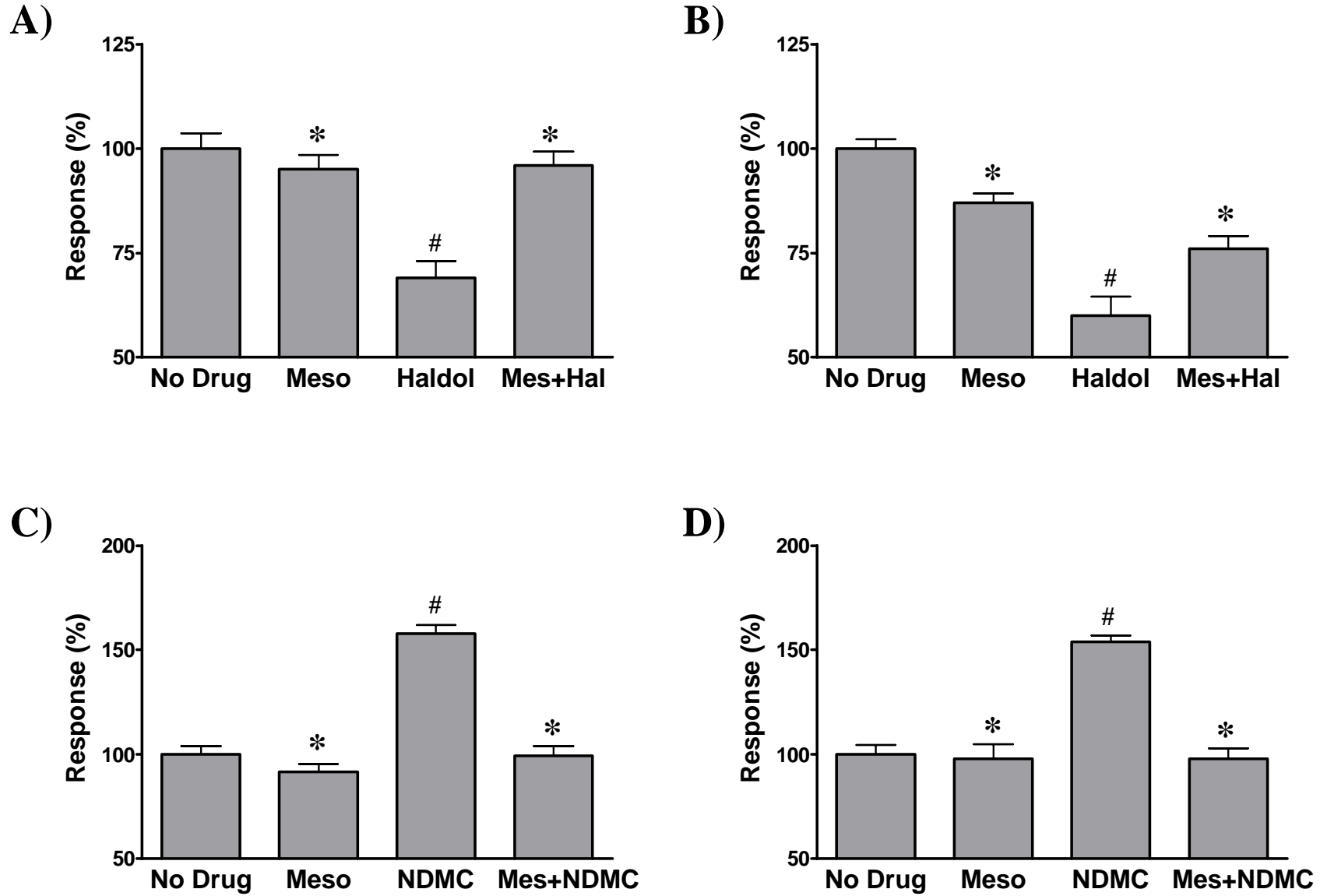
B)



# Figure 2

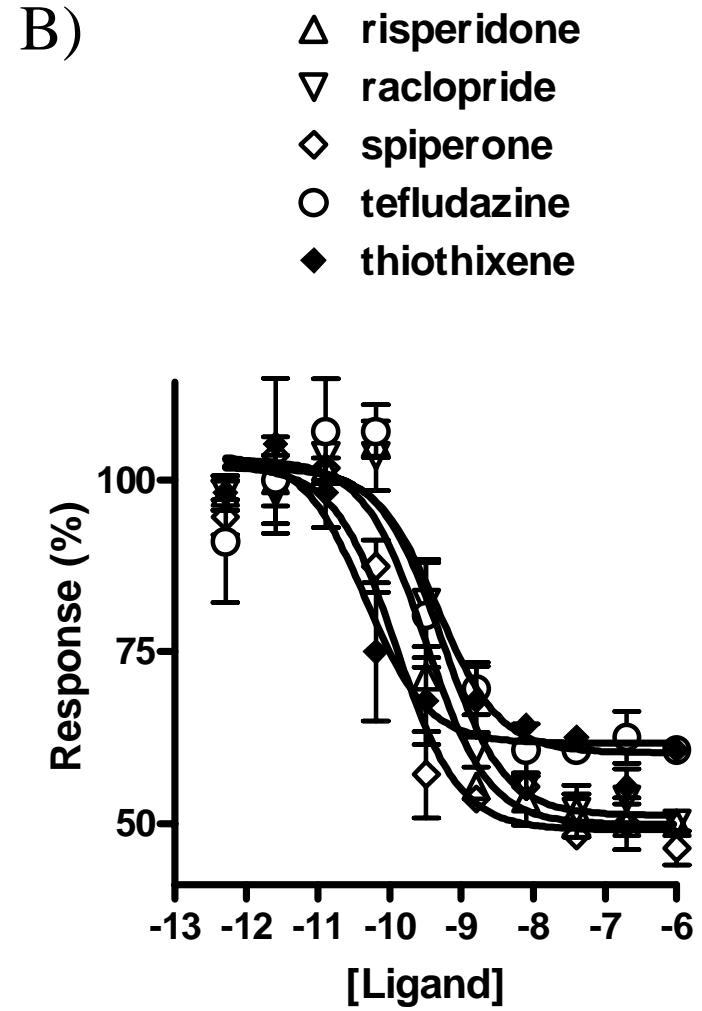
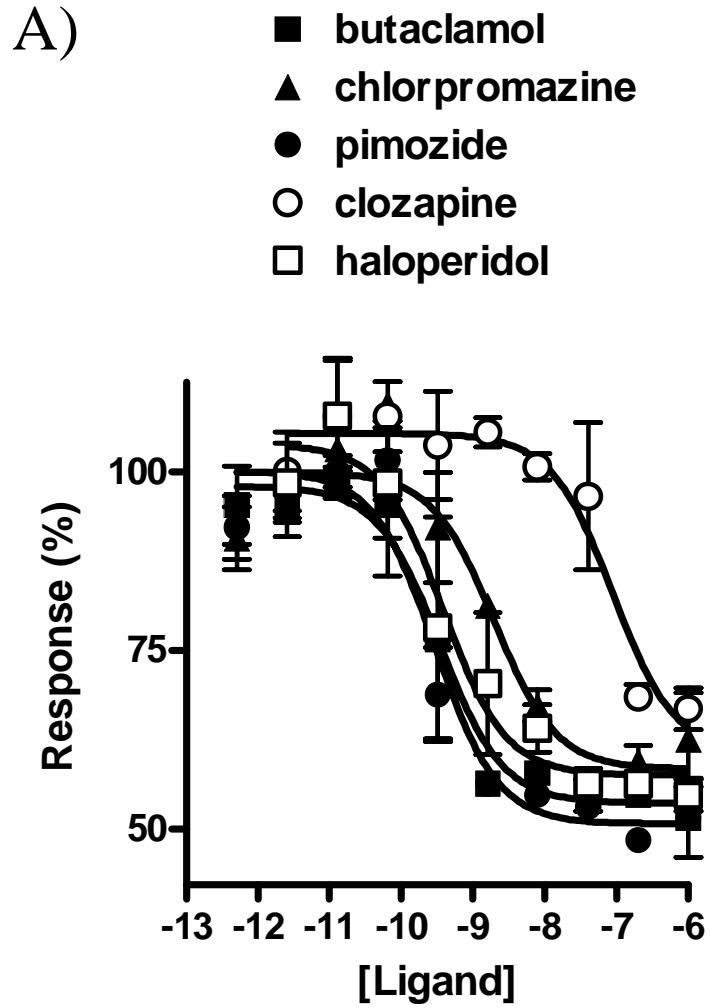


## Figure 3



# Figure 4

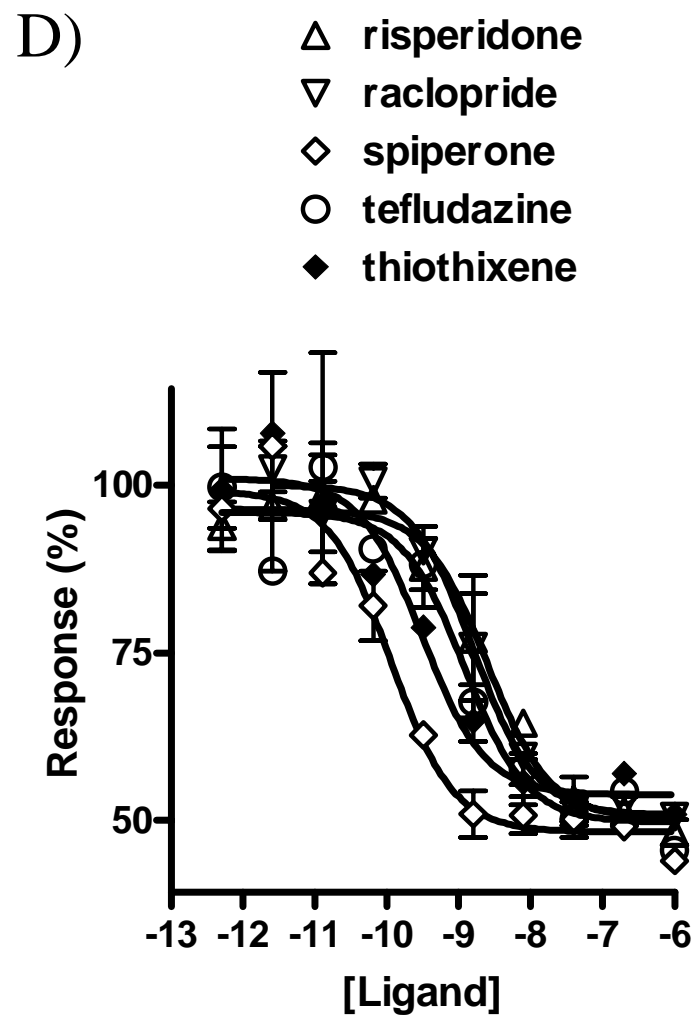
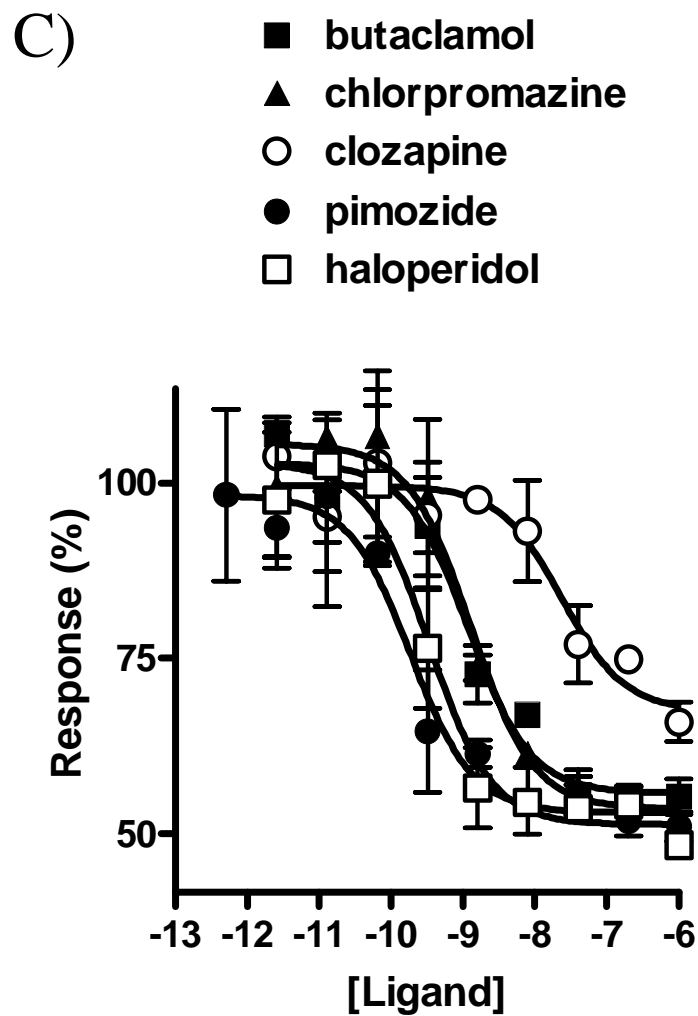
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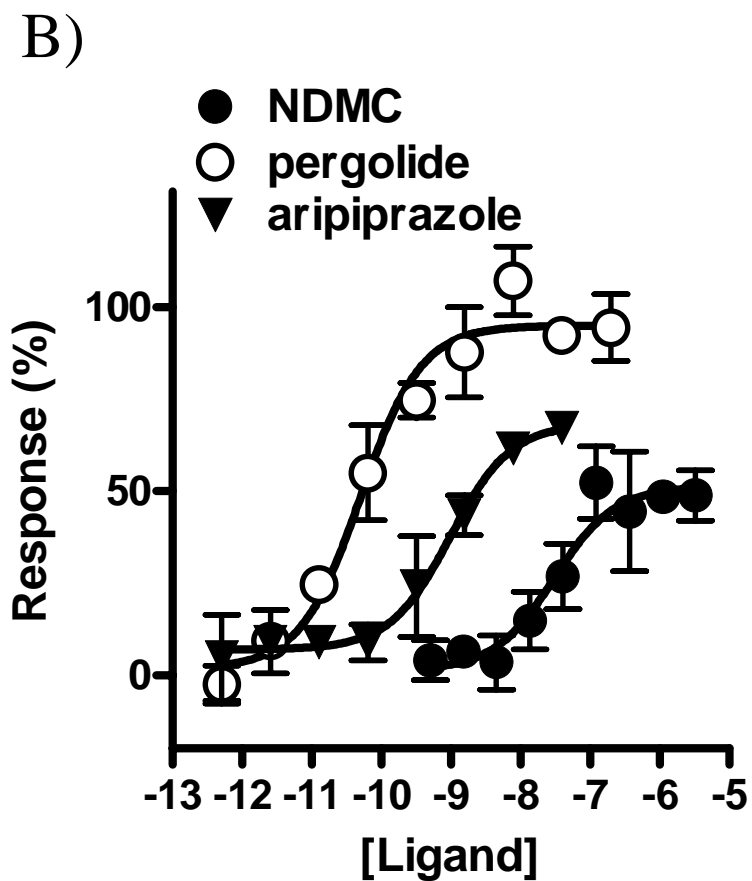
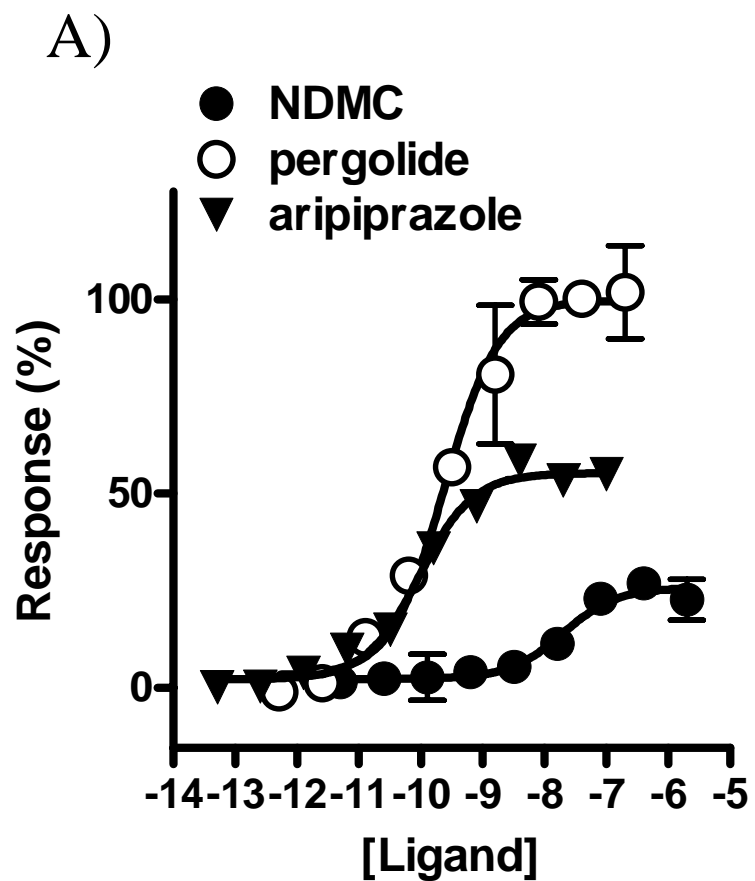
# Figure 4

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# Figure 5

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# Figure 6

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